GENETIC POLYMORPHISM IN CASEINS OF COW'S MILK. END-GROUP ANALYSIS OF β-CASEINS A, B, AND C

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ABSTRACT

The three known genetic variants of β-casein (A, B, and C) have been purified by column chromatography in 3.3 M urea, and their purity verified by polyacrylamide-gel electrophoresis. Qualitative determination of the amino-terminal amino acid by two independent chemical methods revealed arginine for each of the three variants. During reaction with carboxypeptidase A, valine was released most rapidly and was closely followed by isoleucine. The data indicate either an ileu-ileu-val or an ileu-val-ileu C-terminal sequence in each variant. Molecular weights of about 25,000 for β-caseins A, B, and C were calculated from the amount of valine released.

Aschaffenburg (1) has demonstrated that β-casein, second only in concentration to α_{s1}-casein in cow's milk, exists in three genetically determined forms which are termed A, B, and C in order of decreasing electrophoretic mobility. Further, the synthesis of the genetic forms of this protein is breed-specific; β-casein C, for example, has been observed only in the milks of Guernsey and Brown Swiss cows (1, 13).

Mellon et al. (8) carried out amino-terminal end-group analysis on β-casein by dinitrophenylation and concluded that both arginine and lysine were amino-terminal. Dresdner and Waugh (2) studied homozygous β-casein A, and heterozygous β-caseins AB and AC, and observed the presence of isoleucine and valine as C-terminal groups (approximately 2 and 1 moles per 24,000 g).

When the genetic forms of β-casein became available in pure form, an investigation was undertaken to determine the terminal amino acids of these proteins. This paper reports results of that investigation.

MATERIALS AND METHODS

The individual β-casein variants A, B, and C were obtained from individual cows typed homozygous for these proteins. β-Casein fractions were obtained from acid-precipitated casein by the method of Hipp et al. (4). They were purified by twice chromatographing on DEAE-cellulose in 3.3 M urea (14). The purity of each component was verified by polyacrylamide-gel electrophoresis in 4.5 M urea, where single zones were observed for each variant. In the ultracentrifuge, at pH 7.0, $\mu = 0.20$ and 8.5 C, each variant exhibited association behavior (9), although the monomer-polymer distribution differed.

Carboxypeptidase A was obtained as a water suspension of three times recrystallized material from Worthington Biochemical Corporation.[2] The enzyme was treated with diisopropylfluorophosphate (DFP) prior to use.

Carboxyl terminal amino acids. For the determination of C-terminal amino acids, enzymatic hydrolysis with carboxypeptidase A was employed. The β-caseins were dissolved in water at pH 8.2, and the reaction was run in an unbuffered system at 37 C, with a weight ratio of protein to enzyme of 100:1. One-milliliter aliquots of the digestion mixture were withdrawn at specific time intervals ranging from 5 min to 24 hr. The aliquots were precipitated with 0.5 ml of 20% trichloroacetic acid, centrifuged in the cold, and the supernatants decanted and frozen until analyzed. The amino acids released were identified and quantitated by the ion-exchange chromatographic procedure of Piez and Morris (10), using a sample corresponding to 12.05 mg of protein. A Phoenix automatic analyzer was used.[2]

Amino terminal amino acids. The N-terminal amino acids of β-caseins A, B, and C were determined qualitatively by the FDNB (2,4-dinitrofluorobenzene) method of Sanger. The procedures of reaction with FDNB, hydrolysis,

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[2] It is not implied that the United States Department of Agriculture recommends the above company to the possible exclusion of others in the same business.

and identification of DNP-amino acids were essentially those outlined by Levy (3). The DNP-arginine released was also identified, using the modified Sakaguchi reagent (3).

The Edman degradation as applied for paper strips by Fraenkel-Conrat et al. (3) and Schroeder et al. (11) was used to confirm the N-terminal amino acids of the proteins. In addition to the starch-iodide detection method (3), the chromatographic identifications of the N-terminal phenylthiohydantoin (PTH) amino acids were carried out on nonstarched paper, using the Sakaguchi reagent as the color-developing spray.

RESULTS

Figure 1 shows the results of hydrolysis of β-casein A with carboxypeptidase-A. The rate

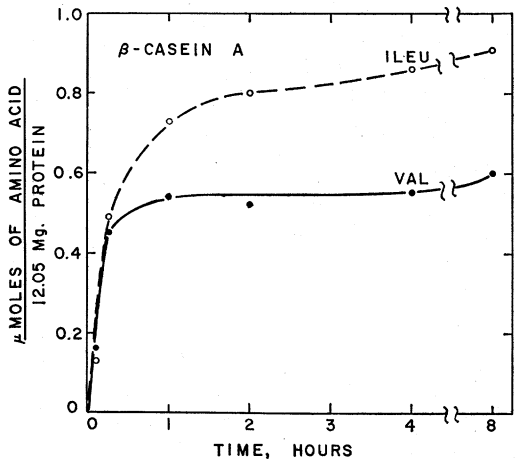

FIG. 1. Action of carboxypeptidase-A on β-casein A. Weight ratio of enzyme to substrate, 1:100; pH 8.2 (unbuffered system); T = 37 C. Aliquot of digest equivalent to 12.05 mg of protein (moisture-free basis) analyzed at indicated times.

of release of amino acids from the B and C variants is essentially identical to that shown in Figure 1. With each variant, valine was liberated first, and was closely followed by isoleucine. This observation is not readily apparent from Figure 1, but is more evident upon examination of Table 1. Over the course of the entire reaction less than 0.15 μmole/12.05 mg protein of the following amino acids was released: leucine, phenylalanine, alanine, and serine. Glutamine was also present and appeared as a chromatographic shoulder on threonine. Because the quantity of isoleucine exceeds that of valine at 60 min, it is suggested that a second isoleucine residue is located close to the end of the chain. The data after 8 hr are consistent with a possible ileu-ileu-val C-terminal sequence, assuming that each variant represents a single polypeptide chain. However, a possible ileu-val-ileu C-terminal sequence cannot be ruled out. Assuming complete liberation of terminal valine, molecular weights of 22,200, 23,800, and 26,600 have been calculated for β-caseins A, B, and C, respectively. If molecular weights are calculated from the amount of isoleucine released after 24 hr of reactions, the values are of the order of 27,000. Longer digestion times are required to fully release this amino acid.

Only arginine was revealed as the N-terminal amino acid in the three β-casein variants by the FDNB procedure. The ether-soluble fraction (3) of the hydrolysis mixture upon chromatography contained only 2,4-dinitrophenol (DNP-OH) and 2,4-dinitroaniline in significant amounts. The DNP-OH was verified by reversible decolorization with acid. Chromatography of the water-soluble fraction (3) disclosed a yellow spot in the position of DNP-arginine. It was positively identified as DNP-arginine and not ε-DNP-lysine by the Sakaguchi reagent. This reagent gave a very strong orange spot for the DNP-arginine control and the DNP-unknowns at exactly the same R_f values. From these data it may be concluded that arginine is one of the N-terminal amino acids of β-caseins A, B, and C. It would follow that if the β-casein variants are single chains, arginine is the only N-terminal amino acid.

When the Edman degradation procedure was applied for identification of the N-terminal amino acids, results of the starch-iodide development were inconclusive. However, after chromatography of the PTH-derivative N-ter-

TABLE 1

Micromoles of amino acid released from β-casein variants (12.05 mg protein) by carboxypeptidase A

Time (min)	β-Casein A		β-Casein B		β-Casein C	
	Val	Ileu	Val	Ileu	Val	Ileu
5	0.16	0.13	0.16	0.14	0.098	0.064
15	0.45	0.49	0.45	0.46	0.45	0.46
60	0.54	0.73	0.50	0.70	0.46	0.71

minal amino acid and PTH-arginine on nonstarched paper using solvent A (3), both gave positive tests for PTH-arginine upon development with the Sakaguchi reagent. Each β-casein variant gave the same result, confirming the FDNB determination. Further, the second step of the Edman degradation procedure strongly suggested that glutamic acid was adjacent to arginine in the N-terminal sequence of all three β-casein variants.

DISCUSSION

According to results of this study each β-casein variant possesses the same amino-terminal and carboxyl-terminal amino acids. The findings of N-terminal arginine agree with the studies of Mellon et al. (8). However, they also reported the presence of 2.4 moles of lysine per 100,000 g of protein as an N-terminal amino acid. This latter finding is presumably the result of differences in preparation of the caseins or, possibly, in the procedures employed for determination of the end groups. These workers also found lysine as N-terminal for α-casein, in addition to arginine. Several workers (5, 7, 9) have determined that arginine is the only N-terminal amino acid in the calcium-sensitive α-casein fraction. The molecular weight values of 22,000, 23,800, and 26,600 for β-casein A, B, and C, respectively, determined from C-terminal valine released, agree within ± 10% of the value of 24,000 reported by Dresdner and Waugh (2). Sullivan et al. (12) and Payens and van Markwijk (9) reported values close to 25,000, based on physical measurements. Presumably, their studies were performed on β-casein preparations which were predominantly β-A, since they used pooled milks (1, 13).

It is evident that the amino acid differences among β-caseins A, B, and C, whether addition/deletion, or substitution must be located within the polypeptide chain if these variants consist of single chains. This observation is similar to that found for α_{s1}-caseins A, B, and C, where C-terminal sequence was leu-leu-tyr and the N-terminal amino acid was arginine (5). Further, it is of interest that both β-caseins and α_{s1}-caseins possess N-terminal arginine. These two proteins are not synthesized independently of each other; that is, the loci governing their synthesis are linked (6).

The data presented do not rule out the possibility that β-caseins consist of two nonidentical chains, one terminating in valine and the other in isoleucine. β-Caseins contain no disulfide which could hold two nonidentical chains intact or have we observed any indication of two zones upon prolonged zonal electrophoresis.

From this report it is concluded that β-caseins A, B, and C possess identical N-terminal residues, arginine, and identical C-terminal sequences, either ileu-ileu-val or ileu-val-ileu. Molecular weight values of about 25,000 are consistent with other chemical data as well as physical measurements. The presence of two nonidentical polypeptide chains of these proteins seems unlikely.

Addendum—Evidence for the close linkage of synthesis of α_{s1} and β caseins has also been presented by Grosclaude, F., Garnier, J., Ribadeau-Dumas, B., and Jeunet, R. 1964. Étroite dépendance des loci contrôlant le polymorphisme des caséines α_s et β. C. R. Acad. Sc. Paris, 259: 1569.

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